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ON THE OCCURRENCE OF COLONY SPECIFICITY IN SOME COMPOUND ASCIDIANS [1]

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In some colonial organisms, colony specificity, or histoincompatibility, has been watched recently by many authors. Colony specificity in some compound ascidians is manifested by the fusibility between colonies; two colonies fuse with each other to form a single mass in one case or do not fuse in the other. This problem was first taken up by Bancroft (1903). According to him, two colonies of different origin in *Botryllus schlosseri* do not fuse together after grafting. Fragments from any one single colony, however, easily fuse together. Sister and brother colonies developed from larvae released by one parental colony sometimes fuse and sometimes do not.

Oka and Watanabe (1957, 1960, 1967) encountered similar results in the Japanese ascidian *Botryllus primigenus,* and the genetic control of colony specificity has been further investigated. The colony specificity in *B. schlosseri* also has been investigated, to a lesser extent, by some authors (Sabbadin, 1962; Karakashian and Milkman, 1967).

In *Botrylloides gascoi* and *B. leachi,* according to Bancroft (1903), fusion is invariably established between two colonies, regardless of their origin. Oka and Usui (1944) have reported that for *Polycitor mutabilis,* in which the zooid of a colony exists solitarily in common test, fusion is never seen when two colonies come into contact as a result of natural growth, but when cut surfaces of two pieces are placed in contact, they can be fused to form a single colony. Hence, in these species colony specificity seems to be absent.

Besides ascidians, the occurrence of colony specificity has been known in some groups of coelenterates, *e.g.*, a hydrozoan *Hydractinia echinata* (Hauenshild, 1954, 1956; Ivker, 1966, 1967, 1972; Toth, 1967) and two anthozoans *Eunicella stricta* and *Lophogorgia sarmentosa* (Theodor, 1970). In certain bryozoans (*cf.* Ryland, 1970, page 30) and freshwater sponges (Rasmont, 1970), the presence of colony specificity has also been suggested.

Thus, the colony specificity seems to be a phenomenon widely distributed among colonial organisms. The present paper reveals the presence of colony specificity in some compound ascidians and some insight is given, from the comparative point of view, into the mechanism involved in the establishment of fusion or rejection and, also, into the evolutionary trend of the colony specificity.

MATERIALS AND METHODS

The materials used in the present experiments were living colonies of five compound ascidians, such as *Botryllus primigenus, Botrylloides violaceus, Pero-*

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phora orientalis, *Symplegma reptans* and *Didemnum moseleyi*. The first three species were collected in the vicinity of the Shimoda Marine Biological Station, Shizuoka Prefecture, the next two in the vicinity of the Usa Marine Biological Station, Kochi Prefecture.

To facilitate handling of the colonies, they were fixed on glass plates. For *Botryllus* and *Botrylloides*, the colonies taken from nature were fastened to glass plates by the method described by Oka and Usui (1944). For *Perophora*, *Symplegma* and *Didemnum*, the colonies, with the substratum or after separation from it, were fastened to glass plates by tying down the edges with string. The plates with the colonies attached were then set in wooden frames and hung below the surface of the sea. The colonies were thus reared in their natural environment, except for transfer to the laboratory aquarium for performing fusion experiments.

The fusibility of the colonies of *Botryllus*, *Botrylloides* and *Symplegma*, was tested by fusion experiments. Fusion experiments were routinely carried out by the following procedure: A piece of colony of about 1 sq cm in size was cut out from each of two colonies. The two pieces were placed in juxtaposition on a glass plate and these colony pieces made a contact with each other. The colonies attached successfully on the glass plates after being kept in a moisture chamber in one hour or so, were returned to the culture boxes in the bay as was just mentioned.

For the *Didemnum* colonies, the above method, to attach them on a glass plate in one hour or so, proved to be a not very practical one. Hence, two colony-pieces with contact were fastened to a glass plate by tying them down with string. The plate was then hung in the bay. The string could be removed the following day. For the *Perophora* colonies, use of the following contrivance was made. That is, two stolon-pieces, each consisting of a stolon fragment one centimeter or more in length and some zooids with the actively pulsating heart, were cut out and placed on a filter-paper sheet in a Petri-dish. They were then forced to be in contact at their cut ends. About twenty minutes later filtered sea water was poured in the dish, when the stolon-pieces, now adhered together in a single stolon, floated on the surface. After removal of the filter paper, the stolon-pieces were submerged to the bottom and cultured in the dish. In each species the fusibility of colonies was examined under two conditions, *i.e.*, by bringing either the cut surfaces or their growing edges into contact.

The observation was made one or two days later under a binocular stereomicroscope. In the reaction resulting from contact of two colonies in respective species, three cases can be distinguished. In this paper, by *fusion* is meant the complete union of blood vessels and/or the test matrix of one colony with the same tissues of the other, in other words, the establishment of a common vascular system or a common test between the two. The case in which some active antagonism is seen between the two colonies is referrd to as *rejection* to distinguish from *indifference* in which no particular reaction can be observed.

Results

Botryllus primigenus

Botryllus is a member of the subfamily Botryllinae (Berrill, 1950). In a colony the individual blastozooids are grouped into star-shaped systems and are connected with one another by the ramifying network of vascular vessels, which terminate in ampullae at the periphery of the colony.

The processes of fusion and rejection have already been described and illustrated in detail by Oka and Watanabe (1967) and Oka (1970). Therefore, the brief account of the processes will be described below.

If the cut surfaces of two colonies were brought into contact, either fusion or rejection occurred. In the case of fusion, both the test and the blood vessels of one colony were united with those of the other to form a single colony. In the case of rejection, after about 24 hours the test cells in the contact area became opaque and slightly brown. This change was easily recognizable with the naked eye as a white line in the contact area between the two colonies.

When contact was made between the growing edges of two fusible colonies, ampullae of each colony mutually extended into the test of the facing colony. By 24 hours or so tip-to-side contacts occurred between facing extended ampullae and there fusion took place, *i.e.,* the blood vessels of the two colonies became interconnected. Both by multiplying the number of fused ampullae and by reducing their size to become internal vessels, finally the original two colonies were completely united into a single colony.

It is a singular fact, as has already been pointed out by Bancroft (1903), that the fusion was never seen between the tips of ampullae. It always took place between the tip and the side of ampullae.

When two non-fusible colonies came into contact at their growing edges, the vascular ampullae of each colony actively extended into each other just like in the case of two fusible colonies. Meanwhile, however, a sign of rejection always appeared at the contact area. The first change detectable was that the ampullae and the test cells in the contact area became deep brown and opaque. Then, about two days after contact, the ampullae detached from the body of the colony and finally disintegrated.

Botrylloides violaceus

Botrylloides is also a member of the Botryllinae and has a common vascular system similar to that of *Botryllus.* Bancroft (1903) could not find the evidence of colony specificity in *B. gascoi* and *B. leachi,* but in our species the presence of it has been confirmed.

If two colonies were placed with their cut surfaces in contact, two fusible colonies easily fused together, but non-fusible ones always rejected each other. In the case of rejection, the blood vessels and the test cells of each colony in the contact area turned black in color as in *Botryllus* and disintegrated in about half a day after contact.

When two fusible colonies were allowed to grow naturally towards each other, ampullae of either colony actively extended into the test of the facing colony until they came into contact with the blood vessels. It was often observed that the tips of the ampullae being in contact with the blood vessels became inflated before fusion took place, as if by contact with the blood vessels the normal growth of the ampullae were being obstructed. Fusion took place after the colonies had been in contact for about a day (Fig. 1A). Also in *Botrylloides,* fusion never occurred between the tips of ampullae in each colony.

In contrast to *Botryllus*, two non-fusible colonies took indifference when they came into contact at their growing edges. Ampullae of both colonies bent upwards and remained pushing against each other for several days, as if they were striving to overcome each other. Usually, however, neither the growth of one colony over the other nor the rejection was recognized. Thus, the edges being in contact grew thicker than usual and a deadlock ensued (Fig. 1B). A colony could readily be pulled off by a pair of forceps from the other, showing that no union of the test-

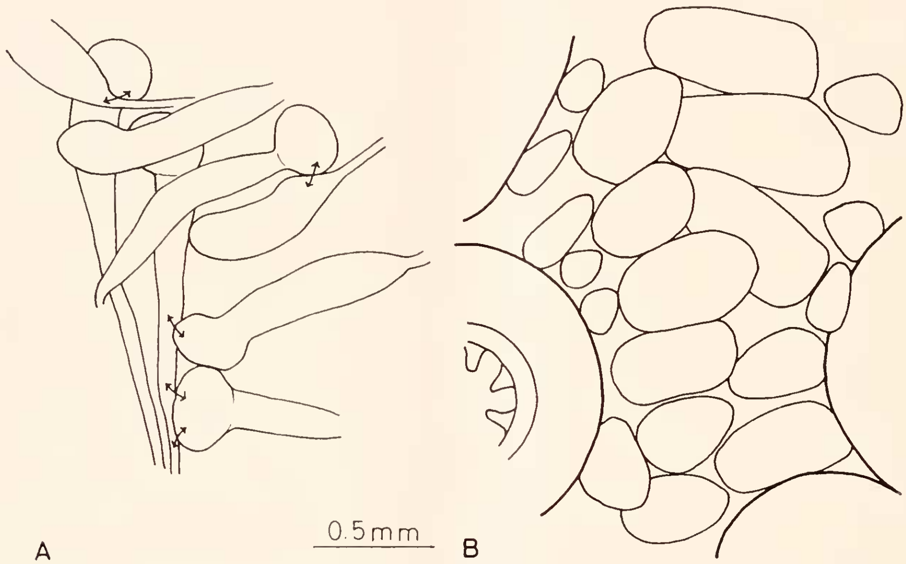

FIGURE 1. Fusion and indifference in *Botrylloides violaceus*. A shows fusion and B shows indifference. In A, arrows indicate the places where the two colonies have fused. In B, two colonies are pushing against each other.

of each colony was accomplished between the two colonies. When they were separated after a long-term contact, however, occasionally a sign of rejection was found in some test cells of the contact surfaces.

Symplegma reptans

Symplegma also belongs to the Botryllinae and has a common vascular system similar to that of *Botryllus*. Among the colonies of *S. reptans*, the presence of colony specificity has been revealed.

When there was contact between the cut surfaces of colonies, either complete fusion or complete rejection took place. The processes of fusion and rejection were essentially similar to those observed in *Botryllus* and *Botrylloides*. In the case of rejection, the blood vessels and the test cells in the contact area gave a necrotic appearance.

When the growing edges of two fusible colonies came into contact, they pushed against each other. In most cases, the ampullae of both colonies remained being in contact at their tips for about a day (Fig. 2A) and finally fusion took place between them (Fig. 2B). As is clear in Figure 2B, in this species tip-to-tip fusion of ampullae was rather common, though of course tip-to-side fusion was also observed in some cases.

In *Symplegma* the ampullae seem to be associated with one another too closely to allow the invasion of the ampullae of another colony. While, in *Botryllus* and *Botrylloides*, in which tip-to-side fusion is usual, the association seems to be too loose to prevent the invasion of the ampullae of other colony. This will be the reason why the tip-to-tip fusion occurs only in *Symplegma*.

When the growing edges of non-fusible colonies came into contact, usually the ampullae of both colonies were pressed tightly against each other as in the case of fusion. About one or two days later, however, the distal parts of ampullae became dark and opaque. Then, the ampullae retracted almost invariably away

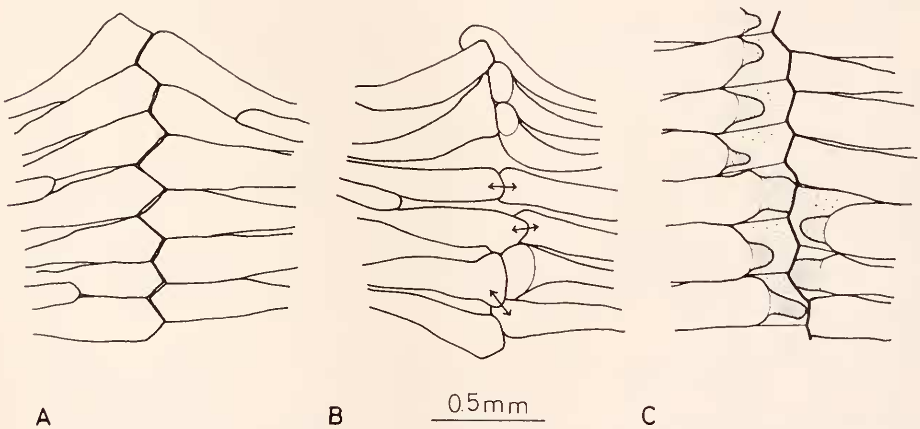

FIGURE 2. Fusion and rejection in *Symplegma reptans*. In A, two colonies are in contact at their growing edges. B shows fusion; fused ampullae are indicated by arrows. C shows rejection. Distal parts of ampullae (densely dotted) have been retracted leaving the empty test (sparsely dotted). The boundary between the two colonies is clearly detected.

from the contact area, leaving there the empty test in which they had formerly extended. In most cases, the boundary of the two colonies was clearly seen under a binocular stereomicroscope. A stage of this rejection is illustrated in Figure 2C.

Before the sign of rejection appeared, each colony being in contact could be easily separated by a pair of forceps from the other. Once it was observed, however, they were no longer separable at the boundary line, thus indicating that the retraction of ampullae was brought about after the establishment of some union between the test matrices of both colonies.

In the process of rejection, the breakdown of ampullae subsequent to their penetration into the test matrix of the other colony, which was the case in *Botryllus,* never occurred in *Symplegma*. In the colonies in which rejection took place, new ampullae always budded at the basal part of the retracted ampullae and continued to grow over them.

The process of rejection presented above is applicable to the combination of colonies of equal strength, armed with the same weapons. If the growing edges of non-fusible colonies, one begin considerably thicker and containing more ampullae than the other, came into contact, the thinner edge was pushed over by the thicker edge instead of being opposed to it. Thus, indifference ensued without giving any evidence of rejection between them. In general, new ampullae just regenerated from the cut ends of blood vessels were less vigorous than the old ones.

Didemnum moseleyi

Didemnum belongs to the family Didemnidae; the colony has no common vascular system in contrast to the above three species. Each zooid is being embedded solitarily in common test and has some vascular processes with faint circulation of blood, which terminate in enlarged bulbs or ampullae. The test usually contains numerous bladder cells, being characteristic of this family. In *Didemnum* also, the presence of colony specificity has been revealed.

If we cut out two pieces from a colony and placed them with their cut surfaces in contact, they fused completely to form a single colony. About 24 hours after contact, the boundary between the original two pieces could not be detected.

On the other hand, rejection resulted from contact between the cut surfaces of two pieces derived from different colonies. The following day after operation, numerous bladder cells existing in the contact area showed an opaque appearance, and the vascular processes had retracted away from that area. Though a union had been established between the test matrices of the two pieces, the boundary line between them could still be detected. Then, at last, the contact area collapsed and disintegrated. Only five colonies were at our disposal, all of which were mutually non-fusible.

When two pieces derived from a single colony came into contact as a result of natural growth, they extended their vascular processes into each other. Thus, at first the two pieces were connected with a thin sheet of test containing vascular processes and later zooids appeared in that area. In the course of this fusion, union between vascular processes never took place.

The pieces derived from different colonies rejected each other when also the contact was made between their normal edges. In the actively growing edges of the colony, vascular ampullae were crowded. When two such edges being non-fusible were placed in apposition, most of the ampullae of each colony always retracted away from the surface shortly before they came into contact, thus giving the impression that the ampullae deprived the colony of its vigor from that area. Some of the ampullae of each colony, however, continued to grow and extended towards each other. After contact, the ampullae and their surrounding test became opaque and finally disintegrated. Two stages of this rejection are illustrated in Figures 3A and 3B.

Perophora orientalis

Perophora belongs to the family Perophoridae, in which respective zooids are connected with one another only by basal stolonic vessels from which they have arisen as buds. The stolons are sometimes branched, but not interconnecting the individuals in a complex vascular network as in *Botryllus*.

From contact between the normal surfaces of two stolons, indifference always ensued regardless of their origin. That is, they continued to grow, adjoining together side by side or crossing one over the other.

When two stolon-pieces cut out from a colony were forced to come into contact closely at their cut ends, they easily fused, *i.e.*, the blood could be distinctly recognized passing from one piece to the other. The same occurred even when two pieces derived from different colonies were used. We used five colonies in all; all of the five colonies were mutually fusible. Thus, two stolons, not only of the same colony but also of differnt colonies, could be easily fused together by grafting. From these results, the conclusion seems to be justified that in a *Perophora* colony specificity is absent.

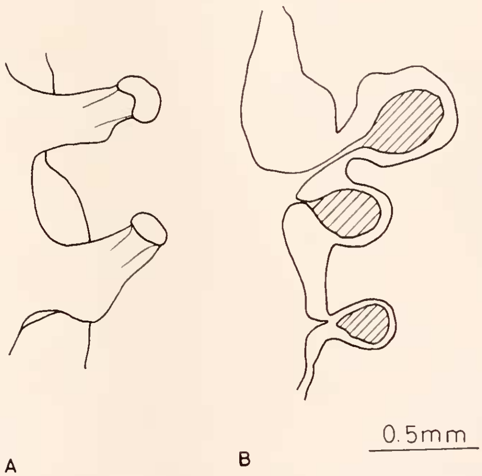

FIGURE 3. Rejection in *Didemnum moseleyi.* In A, two vascular processes of the left-hand colony are extended on the right-hand colony. In B, disintegration occurred in the vascular processes and their surrounding test.

Heterogenetic combinations

Using respective colonies of three species, *Botryllus, Botrylloides* and *Symplegma* belonging to the Botryllinae mentioned before, heterogenetic or interspecific combinations were prepared.

When the cut surfaces of the two colonies of different species were brought into contact, no special phenomenon could be observed between them. In the same way, when the two colonies of different species were allowed to grow towards each other, neither fusion nor rejection was seen to occur between them. They simply competed with each other for the substratum to grow upon. Thus, contact between the two colonies of different species always resulted in indifference.

DISCUSSION

The results from the present experiments, together with those of Oka and Usui (1944) on *Polycitor* colonies, are summarized in Table 1. In the reaction resulting from contact between two colonies, fusion, rejection and indifference are distinguishable. Of these, fusion and rejection are essential; indifference is simply a defect of the other two.

Colony specificity can be considered as a type of allogeneic recognition and manifests itself among others as a hindrance to fusion between two colonies. As is clear from Table I, colony specificity is present in some species, such as *Botryllus*, *Botrylloides*, *Symplegma* and *Didemnum*, but not in *Perophora* and *Polycitor*. According to Bancroft (1903), *Botrylloides gascoi* and *B. leachi* colony specificity is absent, *i.e.*, a union of two colonies is always established either by grafting or by natural growth, regardless of their origin. Discrepancy between the results obtained by Bancroft and the present experiments will be due to the limited number of his observations rather than to variance of species.

TABLE I

Summarizing representation of the results of fusion experiments

Species	Contact between cut surfaces	Contact between growing edges
Botryllus primigenus	Fusion	Fusion
	Rejection	Rejection
Botrylloides violaceus	Fusion	Fusion
	Rejection	Indifference
Symplegma reptans	Fusion	Fusion
	Rejection	Rejection
		(Indifference)**
Didemnum moseleyi	Fusion	Fusion
	Rejection	Rejection
Perophora orientalis	Fusion	Indifference
*Polycitor mutabilis**	Fusion	Indifference
Heterogenetic combinations	Indifference	Indifference

* Oka and Usui (1944).

** When one very vigorous and one rather weak edge come into contact, sometimes indifference ensues.

In those species in which the presence of colony specificity is assured, the growing edges of the same colony fuse together with the complete union of test and/or blood vessels whenever they come into contact. On the other hand, in those species in which colony specificity is absent, when the growing edges or the test surfaces of the same colony are brought into contact, indifference can be recognized. In summary, the presence or absence of colony specificity may be correlated with the ability or inability of fusion between the growing edges of the same colony. Judging from these facts, colony specificity seems to be a feature of common occurrence in those ascidians in which fusion of test and/or blood vessels always occurs between the growing edges of its own. The validity of this assumption in other colonial organisms should be examined by future researches.

When two colonies of different species are brought into either by natural growth or by experimental means, they show indifference to each other. Thus, as has been pointed out by Oka (1970), fusion or rejection recognizable in ascidians is a feature specific within the species, though in certain cnidarians, heterogenetic rejection has been reported (Katô, Hirai and Kakinuma, 1967; Theodor, 1970).

As has been suggested by Oka and Usui (1944) with *Policitor* colonies, the test of ascidians seems to consist at least of two layers, one external and the other internal, the former being thin and tough as compared with the latter. Taking the process of fusion or rejection between the growing edges into consideration, at least two steps will be separated. The first step is the elimination of the external layer of the test, which will be attained enzymatically. The second step is the reaction leading to the completion of fusion or rejection, which itself may consist of a series of reactions.

The first step is either possible or impossible, being alternative. In the second step, however, it seems that the two processes or mechanisms of fusion and rejection are not necessarily alternative; in a sense the former will be more basic and may be overlapped by the latter. In the case of rejection at the growing edges of *Botryllus,* for instance, the two colonies being in contact extend their ampullae into each other as in the case of fusion; before the union of vascular systems takes place, however, the ampullae begin to disintegrate. Judging from these facts, it will be the case that the process of fusion is being interrupted by that of rejection, if it is present. The former is completed only when the latter is absent.

Provided that both the first and the second steps can proceed after contact of tissues, which seems to be the case at least in the botryllids, the following will be justified: In the contact between growing edges, if the first step is lacking the second step is not realized, when indifference ensues. On the contrary, in the contact between cut surfaces the first step is experimentally attained, when either fusion or rejection occurs.

On the basis of the above discussion, the results obtained with respective species will be interpreted below.

In *Polycitor mutabilis* and *Perophora orientalis,* in which colony specificity is absent, the mechanism of the elimination of the external layer is completely lacking. Accordingly, when there is contact between the test surfaces indifference ensues. When there is contact between the cut surfaces, however, fusion occurs, because they are naked, *i.e.,* there is no layer between them.

In *Botryllus primigenus* the elimination of the external layer is always complete when two colonies come into contact at their growing edges, without reference to their relation, fusible or non-fusible. Therefore, in this species the first step is not colony specific. Colony specificity exists only in the second step, and either fusion or rejection is attained.

The results obtained in *Didemnum moseleyi* are similar, seemingly at least, to those of *B. primigenus.* In *Didemnum,* however, most of the vascular ampullae of non-fusible colonies retract away from the surface shortly before they come into direct contact. Only some of them grow upon the other colony and undergo disintegration. These results may be taken to indicate that in this species the substances participating in rejection can pass, to some extent at least, out of the colony. No definite conclusion, however, can be drawn, until suitable studies are carried out.

Botrylloides violaceus will be worthy of particular notice. In this species rejection occurs only when contact is made between the cut surfaces of non-fusible colonies; indifference is always obtained when they come into contact at their growing edges. In contrast to that, when fusible colonies are brought into contact either by means of natural growth or by grafting, fusion invariably takes place Thus, in this species both the first and the second step will be considered as colony specific. It should be noticed here that in *B. violaceus* the specificity of the first step is completely correlated with that of the second step, in other words, fusion and indifference between growing edges perfectly correspond to fusion and rejection occurring between cut surfaces, respectively. How this correlation is governed is an open question.

In *Symplegma reptans* rejection between growing edges takes place only when two colonies remain pushing against each other for a definite period. In this process neither the penetration of ampullae of one colony into the other nor the extinction of the boundary between them is observed. Once any sign of rejection is seen, however, the two colonies cannot be artificially separated from each other indicating the establishment of some union between the test matrices. Since observations on this species are limited, we cannot say indiscreetly whether the elimination of the external layer of the test, *i.e.,* the first step of rejection, is brought about by means of chemical action which may be the case with *Botryllus,* or by means of physical action as a result of pushing against each other. Provided that the latter is the case, both the first and the second step will be colony specific.

More recently, the significance of the colony specificity in relation to the evolution of adaptive immunity of vertebrates has been discussed by Burnet (1971). On the basis of the above discussion and from the view point of the evolution of colony specificity, the colonial organisms may be classified into the following four groups. (1) Colonies have neither the specificity nor the structural barrier to fusion. Some sponges may belong to this group. (2) An external layer which prevents fusion with one another is formed, but the specificity is still lacking. In this group, to which *Perophora* and *Polycitor* belong, the individuality is assured simply by the structural barrier. (3) Specificity in the second step of fusion is acquired. In those species in which the substances participating in rejection can pass through the external layer of the colony or in which no such a layer is differentiated, rejection may take place without colonies coming into direct contact. Such species also, if they exist, should be classified in this group. Thus, *Botryllus* and *Didemnum* belong to this group. (4) Colony specificity is established in both the first and the second steps of fusion. To this group *Botrylloides* and *Symplegma* (?) belong.

It is our present duty to acknowledge our indebtedness to the staff of the Shimoda Marine Biological Station or the Usa Marine Biological Station.

Summary

1. The presence or absence of colony specificity, *i.e.*, the recognition of self and not-self in colonial organisms, has been investigated with several species of compound Ascidians. If the reaction resulted from contact either between growing edges or between cut surfaces of colonies, fusion, rejection and indifference have been distinguished. Of these three cases, indifference means simply a defect of the other two. Both fusion and rejection are specific within the species.

2. The presence of colony specificity has been demonstrated in *Botryllus*

primigenus, Botrylloides violaceus, Symplegma reptans and *Didemnum moseleyi.* But, in *Perophora orientalis* colony specificity is absent.

3. From the above facts, it has been suggested that the colony specificity may be a feature being common to those ascidians in which fusion of test and/or blood vessels always occurs between the growing edges of its own.

4. In the process of fusion or rejection at the growing edges, two steps have been distinguished. The first step is the elimination of the external layer of test, which is followed by the second step terminating in the completion of fusion or rejection. In *Botryllus* and *Didemnum*, only the second step is colony specific. In *Botrylloides*, however, both the first and the second step are colony specific.

5. A possible evolutionary trend of colonial organisms in relation to the colony specificity has been represented.

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